EVALUATION OF STRESS BEFORE, DURING, AND AFTER TRANSPORT IN NAÏVE YEARLING HORSES

A Thesis

by

SHANNON M. GAREY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2009

Major Subject: Animal Science

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Approved by:

Chair of Committee,	Ted Friend
Committee Members,	Dennis Sigler
	Luc Berghman
Head of Department,	Gary Acuff

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ABSTRACT

Evaluation of Stress Before, During, and After Transport in Naïve Yearling Horses.

(May 2009)

Shannon M. Garey, B.S., Iowa State University

Chair of Advisory Committee: Dr. Ted Friend

Recently, the European Union published regulations regarding the welfare of horses during transport requiring that horses be transported in individual stalls separated by partitions. The objective of this study was to determine if concentrations of cortisol, corticosterone, or dehydroepiandrosterone (DHEA) differed among horses with no prior transport experience when transported in individual stalls versus loose groups. Twenty naïve yearlings were assigned to either individual stalls or a loose group, then transported for 6 hours. Ten horses were transported per day (5 in stalls and 5 in a loose group) over a two day trial. The experiment was replicated with a second trial 35 days later, and utilized a switchback design where the horses exchanged treatments between trials. Blood samples were collected and analyzed for cortisol, corticosterone, and DHEA concentrations at pre-transport, 2, 4, and 6 h of transport, and at 2 and 4 h after unloading. After Trial 2, the horses' changed housing from group paddocks to stalls and a follow-up experiment was conducted. The data were analyzed using a mixed model repeated measures ANOVA with the animal as the subject, with trial, treatment, sample time, and treatment-sample time interaction in the model with unstructured covariance

(SAS 9.1). Differences between sample times within each trial, and pre-transport concentrations between trials, were analyzed using paired t-tests (SPSS 12.0.1). No significant differences were found in hormone concentrations for horses transported in individual stalls versus in loose groups. Horses exhibited a significant elevation in cortisol and corticosterone during transport which returned to pre-transport concentrations by 2 hr after transport ($P < 0.01$). Mean pre-transport cortisol concentrations rose significantly in Trial 3 (7.87 ng/ml) from Trials 1 (2.71 ng/ml) and 2 (2.84 ng/ml) ($P < 0.001$). Pre-transport concentrations of DHEA in Trials 1 (482 pg/ml) and 2 (392 pg/ml) also rose significantly in Trial 3 (1607 pg/ml) ($P < 0.01$). Changes in cortisol and DHEA indicated that transportation was a significant stressor for horses, however, being transported in a loose group versus individual stalls was not different. Also, housing changes from paddocks to stalls resulted in significant increases in pre-transport concentrations of stress-related compounds.

DEDICATION

To my amazing husband, Justin, without whom, I would not know the full extent of my potential. His support and faith in me have encouraged me to pick up and go on when I thought I couldn't, and to look back and appreciate what we've done and how far we've come.

To my adoring parents, Bruce and Sherri, whose hearts I broke to move across the country and make this dream a reality. Without the foundation that they provided, I would have never been able to be the strong, independent person that would take a task like this one head on.

Most importantly, to my beautiful daughter Ava, who makes everything in my life worthwhile. She has given me the confidence and compassion to be a better person. She gives me hope, faith and direction in life.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Ted Friend, my committee chair, for the countless hours he spent teaching and guiding me in this project. He is truly a mentor whose patience and wisdom are beyond measure. I'd also like to thank my committee members, Dr. Dennis Sigler and Dr. Luc Berghman. Dr. Sigler, for all of his help in coordinating the horses and schedules to make this project happen, and Dr. Berghman, who made his lab space and time available to help me run and understand the hormone assays that were involved. I am especially thankful to Dr. Josie Coverdale for allowing us to use the research building within the nutrition barn at the horse center while we were transporting the horses, and Dr. Jason Sawyer for helping to keep me going in the right direction when it came to the statistical analysis.

I also want to thank Dr. Penny Riggs for helping us to break into this world of gene expression and for taking her time to guide us along the way. Many thanks also go out to Kelli Kochan who spent a great deal of time and patience teaching me how to work with RNA. For Jamie Butler and her months of trial-and-error work on RNA extraction from blood, I am truly grateful.

I sincerely appreciate Glenda Bingham, Ben Alexander, and Lexie Hayes for their advice and support that have kept me going through this project. I especially thank Sara Tutt for her unwavering dedication to this project and this program. Sara committed countless hours to this project, and for all of her help and support, I am so appreciative.

NOMENCLATURE

h	Hour
min	Minute
sec	Second
d	Day
kg	Kilogram
m	Meter
km	Kilometer
SE	Standard Error
M	Million
CP	Crude Protein
Zn	Zinc
Mn	Manganese
Cu	Copper
Co	Cobalt

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INTRODUCTION

History

Horses have played a vital role in the evolution of American culture. Up until the mid-1900's, horses were a necessity for farm work and travel. They could be found attached to implements, working in fields and attached to buggies, transporting people from farms to town and from one town to another. With the invention of the automobile at the start of the 20th century and the development of tractors in the 1940's, use of horses for farm work and travel was phased out. Over the subsequent half century, people have shifted to using horses for a variety of recreational activities including showing, jumping and racing. Horses went from a vital work animal to a largely recreational activity in less than 100 years. Only a small fraction of the horse population are still used for ranch and farm work. The role they currently play in our society oscillates between livestock and pet, with many regulators finding it difficult to handle the grey area that they represent. Along with the shift in function of the horse came a shift in perspective of how the animal should be treated. As we have progressed from an age of horse-powered transportation, we now find ourselves in an ironic position of transporting those animals for recreational and commercial purposes.

Equine Social Behavior

Horses evolved as a social species living and moving in small groups and rarely

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This thesis follows the style of Journal of Animal Science.

living in isolation. Inclusion within a herd is an essential defense mechanism against predators in a wild or feral living environment. The development of social behavior encourages herd stability by minimizing conflict and establishing a social structure (Goodwin, 2002). Foals spend their first 2 weeks of life bonding and engaging in play activity with their mother. Beyond 2 weeks of age, play interactions with the dam steadily decrease while play and social interactions with other members of the herd steadily increase (Houpt, 1991). The play maneuvers that foals exhibit during this stage of their lives mimic social dominance and mating behaviors that they will use with herd mates as adult horses (Houpt, 1991).

Horses display a social dominance structure within all groups of 2 or more animals. There is clearly a most dominant horse that all others are submissive to and a least dominant horse that is submissive to all others (Waring, 1983). Horses that are not the most dominant, nor least dominant have differing dominance patterns with each other, where all animals have a position in relation to all other animals, although the structure may not necessarily be linear. Dominance is established between two horses during the first few hours of interaction (Waring, 1983). Once a dominance structure is set within a herd, it will remain constant until a member of the herd leaves or a new member joins (Goodwin, 2002).

Horses spend up to 75% of each day grazing as a group (Davidson and Harris, 2002). In the event of a threat, individual animals will stop grazing and move closer to other members of the herd (Waring, 1983). If the threat appears to be a predator and continues to advance toward the herd, the horse closest to it will pivot and begin a flight

response with the other members of the herd immediately following (Waring, 1983). When flight is not an option, horses will use aggression to fight off a threatening individual by biting, kicking with one or both rear legs or striking with one or both front legs. These same aggressive actions are often exhibited by dominant horses toward submissive individuals when they are kept in a high density area where the submissive animal is unable to retreat.

Commercial Livestock

Horses represent a significant portion of the agricultural industry today. Besides livestock sales, horses have a considerable impact on the economy. The total number of horses living in the United States is between 4 M and 9 M, as estimated by the United States Department of Agriculture and the American Horse Council (American Horse Council, 2005, USDA, 2007a). While a majority of these animals are privately owned and used for recreational purposes, a small portion of them are commercial livestock and are being sold for slaughter.

The practice of slaughtering horses in the United States stopped in 2007 due to state laws in Texas and Illinois banning the practice for human consumption; the only states where slaughter plants were operating at the time (Illinois General Assembly, 2007, Texas Statutes Agriculture Code, 1949). However, prior to the closing of those facilities, the United States was processing between 50,000 and 350,000 horses per year (Fig. 1) since the U.S. Department of Agriculture began reporting equine slaughter statistics in 1980 (USDA, 2007b).

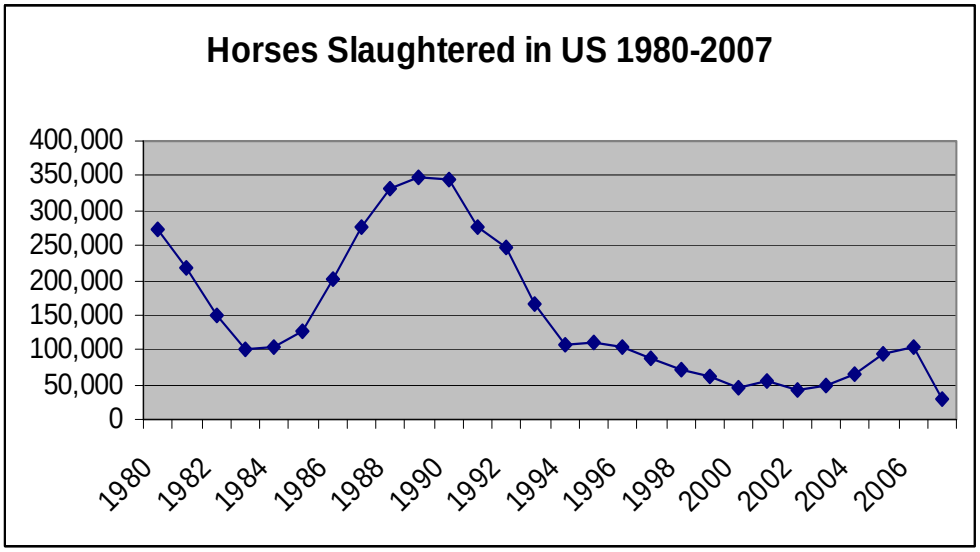

Figure 1. Number of federally inspected horses that were slaughtered in the U.S. between 1980 and 2007 according to the National Agriculture Statistics Service (USDA, 2007b).

In comparison to cattle slaughter facilities, relatively few equine slaughter facilities were open prior to 2007. In the past five years, only 4 plants, located in Texas, Illinois and Nebraska were processing horses, causing many horses that were being transported to slaughter to spend several hours in transit. In addition to the horses being commercially transported to U.S. facilities, many horses were being transported to Canadian and Mexican slaughter plants. In 2007, the year that the last equine slaughter facility closed in the U.S., exports of U.S. horses to Mexico for slaughter jumped over 4 times from about 11,000 horses to over 44,000 horses (Nolen, 2008).

Transport Legislation

In the United States, the 1996 Farm Bill called for USDA regulation of commercial transport of horses. That regulation came in 2002 in the form of 9 Code of Federal Regulations (CFR) Chapter 88. Chapter 88 of the Code bans the transport of commercial horses on two layers, as in a double-deck trailer, which is common in the transport of other livestock species (U.S. CFR, 2002). The same chapter sets out requirements for supplying adequate quality feed and potable water to horses within at least 6 hours of transport, segregating stallions and other aggressive animals during transport and ensuring adequate floor space for each animal. This regulation addresses the physical condition of the horses as well, requiring that the animals transported are fit for travel, are not blind or lame, are able to walk unassisted and are not in danger of giving birth near the time of transport (U.S. CFR, 2002). The final portion of the chapter gives the U.S. Department of Agriculture the ability to fine persons found in violation of the code up to $5,000 per violation, with each improperly transported horse considered a separate violation.

While 9 CFR Chapter 88 set out basic requirements with financial penalties for violations, the Agriculture and Resource Management Council of Australia and New Zealand makes much more specific recommendations to address the animals' physical and behavioral well-being during transport in The Australian Model Codes of Practice for the Welfare of Animals: Land Transport of Horses. This 19 page document focuses with great detail on every aspect of the transport experience, including recommendations on handling to minimize stress, food, water, shelter and rest prior to transport, and even

the condition of loading facilities. More notably, it recommends segregation of horses in stalls during transport using adjustable partitions positioned at a right angle to the direction of travel to reduce the likelihood of injury (ARMCANZ, 1987). In group transported horses, the code suggests that weanlings up to 12 months be allowed 0.7 m^2 per horse, those 12 to 24 months of age be given 0.9 – 1.0 m^2 per horse and adult horses over the age of 24 months be allowed 1.2 m^2 per horse (ARMCANZ, 1987). While this code is much more specific than the 9 CFR Chapter 88 published in the U.S., it is only a set of recommendations, and has no stipulated consequences for failure to comply.

The most recent and most stringent legislation of horse transport has come from the European Union (EU), where, in 2005 The Council of the EU published Council Regulation (EC) No 1/2005 On the Protection of Animals During Transport and Related Operations. Some of the specific mandates included in this document are that all non-registered horses be transported in single layer trailers with a minimum of 75 cm of clearance above the withers and must be offered food and water every 8 hours during transport. The maximum transport period is 24 hours and during long journeys, the horses are to be stalled individually unless they have a foal. Stalls are to be made out of adjustable partitions, and each horse is to be given 1 m^2 to 1.75 m^2 of space depending on age. Horses over 8 months of age are to wear halters and that when tied, they must be able to lie down, eat and drink (Council of the European Union, 2005). In addition to the regulations for transport, the document gives authority to individual member states to ensure compliance and seek out those persons that fail to comply.

In addition to the current codes and regulations, bills have been presented in the U.S. to further regulate equine transport. To date, there have been 2 bills proposed in the current session of Congress that would have a direct impact on the transportation of horses if passed and signed into law. The first was proposed on January 8, 2009 as an amendment to the United States Code Title 49, Transportation, which seeks to eliminate the transport of any horse in a double-deck trailer (U.S. House of Representatives, 2009a). While the regulations set forth in 9 CFR 88 made transport of commercial slaughter horses in double-deck trailers illegal in 2001, this bill would apply that standard to the transport of all horses. The bill has been called the Horse Transportation Safety Act of 2009 and has been referred to the Committee on Transportation and Infrastructure for review (U.S. House of Representatives, 2009a). On January 14, 2009, a second bill was introduced regarding regulation of equine transport. This bill, called The Prevention of Equine Cruelty Act of 2009, is a proposed amendment to Title 18 of the United States Code, Crimes, Chapter 3, Animals, Birds, Fish and Plants. If passed into law, this bill would make transportation of slaughter horses for the purpose of human consumption a criminal offense (U.S. House of Representatives, 2009b). Seven similar versions of this bill have been previously presented to both the House of Representatives and the Senate in past sessions, but have not made it through the legislative process and have expired at the conclusion of each session. The first version was presented to the House in February 2002, and like the versions subsequently presented to the House in February of 2003 and to the Senate in April of 2004, it was referred to a committee for review and never made further progress (U.S. House of

Representatives, 2002, U.S. House of Representatives, 2003, U.S. Senate, 2004). In February of 2005, a fourth version was presented to the House which was reviewed and reported on by the House Agriculture Committee. Two amendments were proposed and rejected, and the bill passed the House vote 263 to 146. From there it was sent to the Senate for review and vote, but failed to progress and, like the previous versions, expired at the end of the session (U.S. House of Representatives, 2005). During that time, a fifth version was presented to the Senate in October of 2005, which was referred to a committee for review, also failed to progress and ultimately expired (U.S. Senate, 2005). A sixth version was presented in January of 2007, and did not advance past introduction. No action was taken and, like the others before it, the bill expired at the conclusion of the session (U.S. House of Representatives, 2007). The most recent version to be presented prior to the current session was introduced to the House in July of 2008 and was reported on by the Committee on Agriculture in September of that year. It also failed to progress from that point and expired (U.S. House of Representatives, 2008).

While the previously mentioned federal legislation is pending, there are several states actively working toward opening new equine slaughter facilities. In late February, 2009, the Montana House of Representatives voted on and passed a bill creating authorization for the construction of an equine slaughter facility and prohibiting any court injunction from delaying such a project. The bill has been sent to the State Senate for review and vote (Montana Legislature, 2009). At the same time, both North and South Dakota have passed legislation in their respective Houses of Representatives to

study the feasibility of opening equine slaughter facilities in their states (North Dakota Legislative Assembly, 2009, South Dakota Legislative Assembly, 2009).

While the future of equine slaughter in the United States is uncertain, the demand for horse meat worldwide remains. That demand, along with the movement of horses for rendering in the U.S. and abroad, ensures that the transport of horses as commercial livestock will continue, and the need for regulation will remain.

Behavior During Transport

As adults, horses within a herd will express dominance only in an effort to control space and avoid conflict (Goodwin, 2002). When space is restricted, the avoidance structure of a herd can become unstable and conflicts may result (Goodwin, 2002). A mathematical model was developed in 1998 to quantify a safe density of loose horses during transport. The model suggested that horses be transported at a density (kg/m^2) equal to 54.837 times the (average animal weight)$^{0.325}$ (Whiting, 1999). The model was based on observations of stocking densities and injuries in foals and mature horses transported to slaughter in 1996 and 1997. Whiting (1999) noted that most horses were transported between 200 and 350 kg/m^2 with only 2 reported injuries and neither required veterinary treatment. In 1999, a study done at the University of California at Davis reported finding fewer injuries of commercially transported horses grouped in a higher density of 1.14 m^2 to 1.31 m^2 per horse versus a moderate density of 1.40 m^2 to 1.54 m^2 per horse (Stull, 1999). However, in the same study, Stull (1999) also reported increased white blood cell counts, total protein concentrations and

neutrophil:lymphocyte concentrations in the higher density group. Stull's findings appeared to concur with a previous study on loading density in cattle transport that concluded that transporting cattle at higher loading densities lowered frequency of movement and heart rates, suggesting that animals utilize the presence of other animals for stability (Eldridge et al., 1988). However, results of a 1992 study comparing cattle transported at high, medium, and low densities clearly indicated that cattle were more stressed under higher stocking densities (Tarrant et al., 1992). The study found that plasma concentrations of cortisol, glucose, and creatine kinase increased as stocking density increased, as did carcass bruise scores (Tarrant et al., 1992). Similarly, a 2000 study of stocking density of horses found that animals transported at a high density of 1.28 m^2 per horse, versus a low density of 2.23 m^2 per horse, were more likely to fall or be injured, and had a decreased the likelihood of rising to their feet after falling (Collins et al., 2000). More recently, researchers analyzed videotaped activity of horses during six 18-20 hour periods of transport in high, medium and low densities. Density had less of an effect on aggressive behavior than did individually aggressive horses that were being transported in each group, but that horses were more likely to fall when transported in high density (Iacono et al., 2007).

A horse's ability to balance during transport is central to its behavior while in transit and may contribute to the amount of stress experienced by the animal. Muscle activity expressed by a horse to maintain its upright position during transport can be used as a direct indication of muscular and emotional stress that a horse experiences as a result of road conditions and driving ability (Giovagnoli et al., 2002). Clark et al. (1993)

studied horses' ability to balance during transport based on forward or rear-facing orientations and found that horses facing away from the direction of travel had fewer indications of loss of balance, however, heart rate and cortisol, used as measures of stress, were not different based on orientation (Clark et al., 1993). A later study by Gibbs and Friend (1999) on orientation and balancing ability found that horses have a slight preference for a 45 degree angle orientation, but that different orientations had no effect on the animals' ability to balance during transport.

Orientation preferences have also been researched in an effort to minimize stress during transport, but with limited conclusions of effect on all horses. As previously mentioned, Clark et al. (1993) reported finding no effects of orientation on cortisol and heart rate measurements, but suggested that the stress induced by transport may have masked differences of orientation. Kusunose and Torikai (1996) conducted a study of the behavior of yearling Thoroughbreds while being transported in loose pairs. They found that when drivers used minimal acceleration and deceleration, the horses spent more time facing away from the direction of travel, however, when drivers made repeated sudden stops, the horses did not show a preference for orientation (Kusunose and Torikai, 1996). Also in 1996, researchers at the University of Edinburgh transported a group of horses in a forward-facing orientation, then reversed to rear-facing orientation for a second transport period. They found that the horses had a slight tendency for lowered heart rates when the horses were forward-facing, but they also found a slight tendency for more frequent movement in the horses when they were forward-facing (Waran et al., 1996). Toscano and Friend (2001) conducted a study placing rear-facing

and forward-facing animals in the same trailer and completing a course of accelerations, decelerations, bumps and turns. The results suggested that, while some horses were better able to maintain balance in one orientation versus the other, the results were mixed for individual animals, and it was concluded that individual factors are more significant than orientation in the animal's ability to balance during transport (Toscano and Friend, 2001).

Dehydration and fatigue can occur after prolonged periods of transport. A previous study conducted by this research group found that horses that had been deprived of water for 6 hours prior to loading were able to be transported for up to 24 hours in hot, humid conditions before becoming severely dehydrated and fatigued (Friend et al., 1998). A follow-up study in 2000 concluded that transport of healthy horses for greater than 24 hours without water caused severe dehydration, while horses transported up to 30 hours with periodic access to water still exhibited increasing symptoms of fatigue (Friend, 2000). A study done at the University of California at Davis concurred that when horses are transported up to 24 hours, they exhibit symptoms of dehydration, depressed feed and water intake and post-transport weight loss. Physiologically, this study also showed that the horses' packed cell volume increased, along with cortisol concentrations during the transport period (Smith et al., 1996). Researchers followed-up this study in 2000 by evaluating indices of stress in horses transported for extended periods during hot summer conditions. They reported that after 24 hours of transport, the horses lost weight, exhibited symptoms of dehydration, had

increased concentrations of lactate, glucose and cortisol, and showed an increased neutrophil:leukocyte ratio (Stull and Rodiek, 2000).

In an attempt to alleviate the stress of transport on horses, researchers at the University of Bristol studied the effects of blindfolding the animals during handling and loading into transport vehicles. They evaluated the horses' heart rates and behavior and concluded that blindfolding increased the animals' stress levels and made them more difficult to handle (Parker et al., 2004).

Indices of Stress

As one can see from the numerous studies conducted on transport stress in horses, there are many ways to identify stress in animals. Behavioral indicators of stress that have been measured during transport include number of movements, number of falls, number of aggressive acts toward other horses, position of the animal's head and amount of eating and drinking. In addition, researchers have used a variety of physiological measures such as electromyography of muscle activity, heart rate, respiratory rate, body temperature, weight, white blood cell count, red blood cell count, packed cell volume, neutrophil:lymphocyte ratio, and concentrations of ACTH, cortisol, glucose, lactate, plasma protein and creatine kinase. While many of these measures give an indication of stress in an animal, no one measure has been shown to definitively quantify stress.

General Adaptation Syndrome. In the mid-1940's, Hans Selye described what he called the General Adaptation Syndrome, which encompassed three stages of a stress

response in animals and humans. He theorized that in the first stage, called alarm, when a stressor is presented, the pituitary is stimulated to express adrenocorticotropic hormone (ACTH) which causes the adrenal cortex to produce glucocorticoids and mineralocorticoids, which travel throughout the body to target organs (Selye, 1951). In this stage the sympathetic nervous system is engaged through increased heart rate, blood pressure and respiratory rate, supplying the body with increased blood flow and oxygen. Cortisol, a glucocorticoid, stimulates liver cells to perform gluconeogenesis and subsequently, blood glucose concentrations rise supplying the body with higher energy levels. In the second stage, adaptation, the initial hormone response subsides, while the body continues to resist the stressor and the immune system becomes suppressed. Finally, in the exhaustion stage, the body has depleted its resources and stops resisting. In extreme cases, the exhaustion stage results in death.

Suppressed Immune Function. Respiratory disease has been one of the biggest concerns of transporting livestock for many years. Many animals that appear to be in good health when loaded for shipment begin to express symptoms of respiratory disease within hours of being unloaded after transport.

Researchers at Kitasato University in Japan reported in 1995 that after studying young Thoroughbreds that were transported for approximately 41 hours, transport predisposes the upper respiratory tract and lower airways of horses to insult from pathogens, which can lead to respiratory disease and potentially, pneumonia (Oikawa et al., 1995). Results of a subsequent study by the same research group suggest that transporters who increased rest periods and cleaned out the trailer during those periods

could reduce overall transport stress on the horses as well as reduce airborne respiratory irritants (Oikawa et al., 2005).

Cortisol. When studying stress, glucocorticoid concentrations in the blood can give researchers an indication of an individual's reaction to a given stressor. As early as 1960, researchers were correlating corticosteroids with stress response. Parkinson et al. (1960) determined that there were differences in corticosteroid concentrations between European and Bantu patients which they theorized contributed to differing stress reactions after surgical procedures. By the mid-1970's researchers at Bucknell University had demonstrated differences in cortisol concentrations between dominant and submissive squirrel monkeys when exposed to a series of stressors (Manogue et al., 1975). Later research directly linked psychological stress to increased cortisol concentrations when a study comparing military parachutists before and after their first two jumps from an airplane, found significant increases in cortisol, prolactin and thyrotropin (Schedlowski et al., 1992). Cortisol does not, however, correlate linearly to stress experienced over long periods of time. A 6 d study of military cadets on a deprivation diet with only 1 – 3 h of sleep per day that were subjected to intense physical training activities found that cortisol peaked on d 3 and was returned to near basal levels by d 5, although the cadets remained under intensely stressful conditions until d 6 (Opstad, 1992). However, cortisol is commonly used to identify stress, especially short-term stress, in human and animal research studies.

DHEA. A hormone that has recently been used in the field of stress research is dehydroepiandrosterone (DHEA). DHEA is a neurosteroid produced by the adrenal

glands. Researchers at the Technical University in Munich, Germany have recently determined that severely depressed patients exhibited increased diurnal DHEA concentrations over healthy control patients when evaluated every 30 min for 24 hours (Heuser et al., 1998).

As with cortisol, DHEA secretion is initiated by ACTH, although differences between DHEA and cortisol expression have been observed and are not fully understood, and some researchers are using DHEA:cortisol ratio as a physiological measure in their studies (Scott et al., 2000, Yehuda et al., 2006). Scott et al. (2000) demonstrated a difference in DHEA:cortisol ratio over time between chronic fatigue syndrome patients and healthy control patients. The researchers administered ACTH to both groups and evaluated the subsequent plasma DHEA and cortisol concentrations. While the control patients exhibited a decrease in the ratio, the patients with chronic fatigue syndrome did not (Scott et al., 2000).

A post-traumatic stress disorder (PTSD) study conducted at the Mount Sinai School of Medicine found that veteran patients with the disorder had significantly higher DHEA concentrations than veteran patients without PTSD (Yehuda et al., 2006). Additionally, researchers working on that study were able to show a correlation between DHEA concentrations and severity of the disorder symptoms. They were also able to demonstrate a correlation between cortisol:DHEA ratio and the severity of childhood trauma, as well as PTSD symptom severity (Yehuda et al., 2006).

While there is still a lot of information about DHEA that is unknown, these early studies indicate that there may be a link between long-term stress disorders and elevated

concentrations of DHEA or increased DHEA:cortisol ratios. If DHEA can be shown to be an indicator of long-term stress, researchers may be able to identify and change stressors in animal production that lead to impaired growth or performance.

Objectives

The objectives of this study were to determine if differences in physiological stress markers exist when horses are transported in individual stalls versus being transported as a group. Further, the study sought to determine if horses continue to exhibit elevated stress markers after the termination of a 6 h transport period. Stull and Rodiek (2002) concluded that when horses were transported for long periods of time, those that were cross-tied exhibited greater indications of stress and took longer to return to pre-transport measurements.

Additionally, this study sought to determine if changes in housing and exercise patterns have an effect on the response of horses during transport.

Hypothesis

It may be hypothesized that, due to the social behavior of horses, animals may exhibit decreased physiological indicators of stress when transported in a group, as opposed to enduring the stress of transport in an isolated stall. This researcher hypothesizes that termination of transport, the stressor, will result in a quick reduction of glucocorticoids to pre-transport concentrations as the animals cease the resistance stage of adaptation.

MATERIALS AND METHODS

Horses

Twenty yearling quarter horses that had no prior hauling experience were selected from the herd at the Texas A&M University Horse Center. All of the yearlings selected were simultaneously participating in a nutrition and exercise physiology study examining the influence of dietary supplements on the incidence of gastric ulcers. All horses were receiving 1.25% of body weight of CP textured grain mixture, 1% of body weight of hay as their basal diet and one of three supplement treatments. Seven horses were receiving a sulfated form of trace mineral containing Zn, Mn, Cu, Co and Calcium carbonate added to the grain mixture at 41.6 mg/kg of body weight. Six horses were receiving a proteinated form of trace mineral containing Zn, Mn, Cu, Co and Calcium carbonate added at 41.6 mg/kg of body weight. The remaining seven horses were given no supplement to their daily grain and hay diet. Horses were fed twice a day for 3 h per feeding period. During feeding periods, horses were placed in assigned feeding stalls within the nutrition barn at the Horse Center, and stall assignments remained consistent throughout Trials 1 and 2. Between feeding periods, the horses were housed in group paddocks around the perimeter of the nutrition barn. Group paddocks were assigned by feed treatments, such that all horses within a feed treatment shared a paddock. While at the Horse Center, horses were exercised 3 d per week for 20 min per d, by being enclosed in an automated equine walker.

All horses were assigned a temperament score of 1-5 based on the primary caretaker's experiences and interactions with each animal over the past year. The horses were then blocked by temperament score and diet, and assigned to one of two treatment groups for transport; tied in individual stalls or loose group.

Transport and Treatments

Three trials were conducted in which each horse was transported for 6 h. Each trial was completed over a 2 day period and there were 35 days between trials. Five horses from each treatment group were transported on the first day and the remaining five horses in each treatment group were transported on the second day. Using a switchback design, the treatment that each horse was exposed to in the first trial was reversed for the second trial. Trial 3 was conducted as a follow-up in which each horse was exposed to the same treatment they received in Trial 1.

The study was carried out using a custom built 16.2-m long x 2.4-m wide x 2.62-m high, single-deck, slat sided trailer (Barrett Trailers, Purcell, Oklahoma) pulled by a tractor. The trailer was divided into three sections: individual stalls, a loose group compartment and a small staging area for sample collection in the center (Fig. 2). At the conclusion of each trial day, the placement of the stalls and group compartment was reversed within the trailer to mitigate any confounding effects of traveling in the front versus the rear portion of the trailer. In addition, the direction of the stalls was reversed to mitigate any confounding effects of position or air flow when facing the passenger side versus the driver side of the trailer (Fig. 3).

Figure 2. Photograph of the tractor and trailer in which the horses were transported. The vertical support posts that were spaced at 0.914 m intervals and were used to attach stall partition panels and group compartment gates are visible from the outside. Traction bars on the floor of the ramp can also be seen.

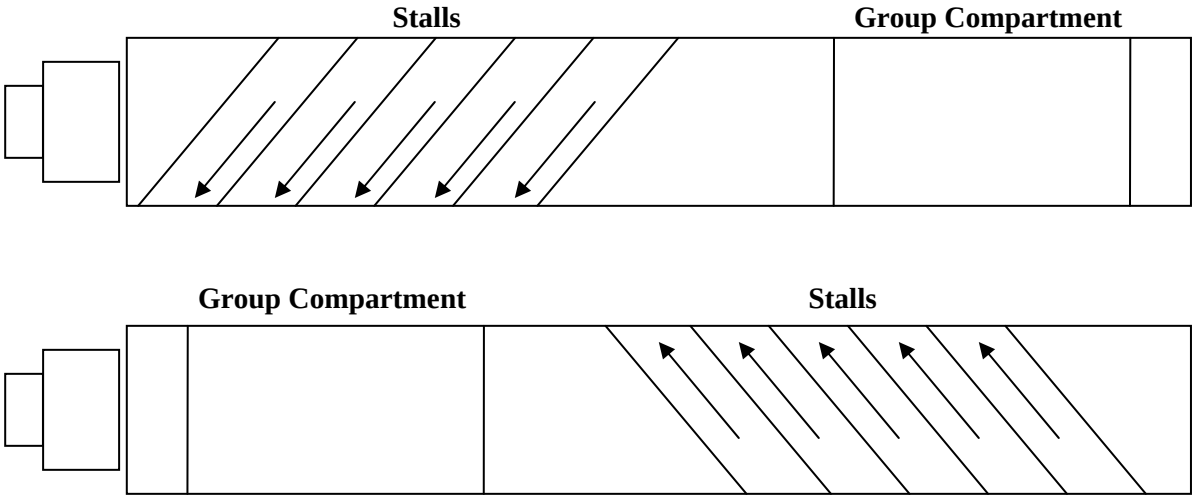

Figure 3. Floor diagram of the trailer showing stall and loose group placement during the first (top) and second day (bottom) in Trials 1 and 3. Configuration was reversed in Trial 2, such that loose horses were in the front of the trailer on the first day (bottom) and in the rear on the second day (top).

All horses were haltered during transport, and the stalled horses were tethered with a 0.46 m trailer tie made of bungee. The trailer ties were connected by a bull snap to the frame of the trailer approximately 0.2 to 0.3 m above the horses' topline, and connected, below the jaw, to the O-ring of the horses' halters by a quick-release clip.

Loading and Unloading. The horses were loaded and unloaded using a portable livestock loading ramp with semi-solid sides and evenly spaced floor bars for traction. Gates were placed from the door of the barn to the ramp in a funnel design to encourage forward movement in the direction of the trailer (Fig. 3). Because the horses used in this study had no previous transport experience, all horses were conditioned to loading and unloading 3 d prior to the start of Trial 1. During this conditioning phase, each horse was gently encouraged to enter the ramp and the trailer without force. Once inside, all horses were placed in an individual stall and tethered to a trailer tie for less than 1 min, while the stall was closed. The horses were then lead back to the ramp and allowed to unload at their own pace.

Stalls. Five individual stalls were constructed by diagonal placement of six 3.048 m utility panels (Priefert, Mount Pleasant, TX) at 0.914 m intervals, with each panel attached to a support post of the trailer by a, $^{3}/_{16}$ inch chain and galvanized smooth wire (Fig. 4). The panels were elevated such that the lower bar was 0.914 m off the floor of the trailer to prevent the horses from getting hooves or legs entangled in any open portion of the partitions. Once horses were loaded into each stall, the partition was closed and a set of $^{3}/_{16}$ inch chains were used to attach the panel to the opposite wall of the trailer. Each stall encompassed 2.79 m^2, although the horses were not able to utilize the entire space because of the diagonal presentation of the stalls and because they were tethered. Because the horses were tied, the portion of the stalls to which the horses had access was 0.711 m by 2.032 m, which gave each horse 1.44 m^2 of floor space. This resulted in an average of 263 kg/m^2 per horse for the first trial, 279 kg/m^2 per horse for the second trial, and 287 kg/m^2 per horse for the third trial, given the inter-trial growth of individual horses.

Figure 4. Photograph of diagonal stall design and placement within the trailer, showing elevated partitions and how each horse was loaded and unloaded.

Group Compartment. The group compartment was constructed using the two 2.54 m solid swing gates that were manufactured specifically for the trailer. One gate remained in the factory installed mounting, while the second gate was positioned at the support post that best yielded the desired amount of floor space. This design allowed the gate on the factory installed mounting to swing freely and latch securely. The second gate was firmly attached using four $^{3}/_{16}$ inch galvanized chains at both top corners and around the central part of the gate on each side. The lower part of the gate was secured using galvanized smooth wire applied in approximately 8 layers. In Trial 1, the movable

gate was placed 2.29 m away from the factory installed mounting and for Trials 2 and 3, it was placed 2.74 m from the first gate to allow for inter-trial growth of the horses. There was a total of 5.82 m^2 of floor space in Trial 1, and 6.96 m^2 of floor space in Trials 2 and 3. In Trial 1, the loose horses averaged 325 kg/m^2 of floor space, while in Trials 2 and 3, the horses averaged 284 kg/m^2 and 299 kg/m^2 of floor space respectively.

Sample Collection

A pre-transport sample was drawn 15 min prior to loading. Additional samples were drawn after 2, 4 and 6 h of transport while the horses were still inside the trailer, and two final samples were collected at 2 and 4 h after unloading. To avoid scar tissue buildup from multiple collections at the same site, horses were marked at 6 different sites along the jugular vein prior to the start of each trial. Blood samples were collected using 20 gauge 1½ inch needle with holder (Vacutainer® Becton, Dickinson and Company, Franklin Lakes, NJ) and 9ml plastic evacuated collection tubes containing sodium heparin (Vacuette, Greiner Bio-One, New York, NY). Immediately after each sample was collected, the tubes were inverted multiple times and placed on ice until the total collection was complete. Sampling time ranged from 20-30 minutes per collection for all ten horses.

For collection of the pre-transport sample and those drawn after unloading, each horse was haltered in its feeding stall and one researcher used a fluttering motion over the horse's left eye for distraction while the second researcher applied pressure to the left jugular vein for 5-25 sec, inserted the needle and attached the evacuated tube. The order of collection followed the order in which the horses were stalled. However, when the horses were loaded in the trailer, the stalled horses were collected in the reverse order that they were loaded, and the group horses were collected in the order that they were caught when the researchers entered the compartment. Due to the secure attachment of the stall partitions, stalls were not opened to access the horses for blood sampling during transport. Instead, the researchers entered each stall by ducking under each panel and untying the horse from the trailer and backing it into the unoccupied portion of the stall. After the sample was collected, a researcher would remain with each horse to keep them restrained while samples were collected from the remaining horses (Fig. 5). Once all samples were drawn, the researchers returned to each stall, reattached the horses to the trailer using the trailer tie and then exited to the central staging area of the trailer.

Figure 5. Photograph of the in-stall sample collection showing horses being restrained by one researcher while another ducks under a partition to get to the next horse. Also shown is the height at which each horse's head was tied and the chains and wire that held each partition to a support post of the trailer.

Once all horses had been sampled, the tubes were again inverted multiple times to ensure thorough mixing. In Trials 1 and 2, the tubes were then processed through a leukocyte capturing filtration system (LeukoLOCK^{TM} Total RNA Isolation System, Ambion, Inc., Austin, TX) that collected the filtered blood in a glass evacuated collection tube with no chemical additives (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ). The filters were further processed by adding an RNA preservative (RNAlater, Ambion Inc., Austin, TX) and stored at -20ºC. The filtered blood was then centrifuged at 3300±165 rpm for 10 min and the plasma supernatant was collected into 5 ml snap-cap tubes and stored at -20ºC. During Trial 3, the blood samples were not filtered for leukocytes, but were immediately centrifuged for plasma collection as previously described.

Housing Change

Five days after the conclusion of Trial 2, all 20 horses were moved from the Texas A&M University Horse Center to the Texas A&M University Freeman Arena. The horses switched from group paddock housing at the Horse Center to being stalled individually in solid-wall stalls at Freeman Arena. They were exercised for at least 15 min per day, 3 days a week. For the first half of the period that the horses were housed at Freeman Arena, 10 of them were involved in an undergraduate class where they were lounged for 15 min per day, as well as being handled and groomed for an additional 45 min per day, 5 days per week. The remaining 10 horses were lounged for 15 min per day, 3 days per week and were turned out in groups of 2 for 45 min per day, 2 days per

week. For the second half of the period that the horses were housed at Freeman Arena, the exercise treatments were reversed.

Hormone Assays

Plasma samples that were stored at -20°C were analyzed by colorimetric ELISA for cortisol, corticosterone and dehydroepiandrosterone concentrations. Complete ELISA kits were used for all assays (Assay Designs, Ann Arbor, MI). A steroid displacement reagent was added to cortisol and corticosterone samples before testing to facilitate measurement of total hormone concentrations as opposed to free hormone concentrations. Protocols for each kit were followed exactly as written. Samples were run in duplicate and plates were read at 405 nm optical density on a plate reader (Wallac Victor II 1420, Perkin Elmer, Waltham, MA). Known concentration standards were used on every plate to optimize comparisons between plates. Data obtained from the plate reader were inputted into a curve-fitting software program (StatLIA®, Brendan Technologies, Inc., Carlsbad, CA) that calculated total concentrations of each hormone based on averages of the duplicate samples in comparison to the generated logarithmic curve of the known standards.

RNA Extraction

Leukocytes that were captured by filtration were rinsed with Phosphate Buffered Solution to remove the RNA preservative that was added during sample collection. The filters were then flushed with a cell lysing reagent and the genetic material infused

product was collected into a 15 ml centrifuge tube. Further extraction of the RNA from the liquid followed the protocol included with the complete filtration and extraction kit (LeukoLOCK^{TM} Total RNA Isolation System, Ambion, Austin, TX). Within the protocol, RNA was bound to beads and washed repeatedly with isopropanol. A DNase reagent was applied to the samples to degrade any DNA that may have been present and contaminating the product. The final RNA product for each sample was eluted from the beads back into solution using 50 µl of an elution solution and stored at -80ºC.

RNA Analysis

A preliminary analysis of four samples was done on a 384-well Real-Time Polymerase Chain Reaction (PCR) plate coated with primers for 84 human genes that are involved in the inflammatory response (SuperArray, SABiosciences, Frederick, MD). Some of the primers used on the plate included chemokines, interleukins and members of the tumor necrosis factor family (Appendix Table 12). Samples were analyzed that were taken pre-transport and after 4 h of transport to compare effects of transport. Additionally, samples chosen for analysis were selected for their differences in cortisol concentrations. One horse was selected that exhibited relatively low cortisol responses and another horse was selected that exhibited relatively high cortisol responses when comparing all horses at every sample time. The pre-transport and after 4 h of transport samples from each horse were thawed on ice for 30 min. The RNA was converted to cDNA and added to the manufacturer's master mix by following the protocol included

with the plate. The cDNA that was added to the plate was then amplified in a fast real-time PCR system (ABI 7900HT, Applied Biosystems Inc., Foster City, CA).

Statistical Analysis

Hormone Concentrations. Treatment effects on the plasma concentrations of cortisol, corticosterone and dehydroepiandrosterone (DHEA) during Trials 1 and 2 were analyzed using a mixed model repeated measures analysis of variance with each individual as the subject, with trial, treatment, sample time, and treatment-sample time interaction in the model with an unstructured covariance (SAS 9.1, SAS Institute, Inc., Cary, NC). Differences between sample time periods were analyzed using paired T-tests within each trial (SPSS 12.0.1, SPSS Inc., Chicago, IL). Least squares means are presented where the data have been adjusted for covariance.

Treatment effects in Trial 3 were analyzed separately from the first two trials because of the change in housing and exercise programs. Hormone concentrations were analyzed using a mixed model repeated measures analysis of variance with each individual as the subject, with treatment, sample time, and treatment-sample time interaction in the model with an unstructured covariance.

Paired samples T-tests were used to compare pre-transport plasma concentrations of each hormone between the three trials (SPSS 12.0.1, SPSS Inc., Chicago, IL). The pre-transport pooled means were compared without regard to treatment because there was no treatment applied prior to transport.

A mixed model repeated measures analysis of variance with each individual as the subject, with trial, treatment, supplement, sample time, and treatment-sample time interaction in the model with an unstructured covariance was used to determine if there were any differences in hormone concentrations based on dietary supplement in Trials 1 and 2. The same procedure was used to analyze the concentrations of each hormone observed in Trial 3, with trial omitted from the model.

RNA Analysis. Gene expression data from the real-time PCR plate were considered qualitative due to the lack of representation of treatments and replicates. In further studies, the data collected from Real-Time PCR analyses will be quantified and statistically analyzed.

RESULTS

Trials 1 and 2

Cortisol. Transport in individual stalls or in a loose group did not have a significant effect on cortisol concentrations in Trials 1 and 2 (Table 1). There was no significant interaction between treatment and sample time ($P = 0.371$) and there were no statistically significant differences in cortisol concentrations between treatments ($P = 0.713$). Pre-transport concentrations were significantly lower than concentrations after 2 h, 4 h and 6 h of transport, but did not differ significantly from concentrations at 2 h and 4 h post-transport ($P < 0.01$).

Table 1. Least squares means of cortisol concentrations (ng/ml) in relation to time relative to start of transport for both treatment groups during Trials 1 and 2.

	Treatment		
Time	Stall	Group	SEM
Pre-transport	2.74[a]	2.81[a]	0.88
Transport			
2 h	19.31[b]	16.98[b]	3.24
4 h	20.25[c]	18.82[b]	3.80
6 h	16.55[b]	15.01[c]	3.14
Post-transport			
2 h	4.31[a]	4.27[a]	1.56
4 h	3.87[a]	2.45[a]	0.82

[a,b,c] Means within a column without a common superscript differ ($P < 0.01$)

Corticosterone. Transport in individual stalls or in a loose group did not have an effect on corticosterone concentrations in Trials 1 and 2 (Table 2). There was no

significant interaction between treatment and sample time (P = 0.470) and there were no statistically significant differences in corticosterone concentrations between treatments (P = 0.370). Pre-transport concentrations were significantly lower than concentrations after 2 h, 4 h and 6 h of transport, but did not differ significantly from concentrations at 2 h and 4 h post-transport (P < 0.01).

Table 2. Least squares means of corticosterone concentrations (ng/ml) in relation to time relative to start of transport for both treatment groups during Trials 1 and 2.

	Treatment		
Time	Stall	Group	SEM
Pre-transport	5.29[a]	3.35[a]	1.17
Transport			
2 h	17.91[b]	13.74[b]	2.92
4 h	14.11[b]	13.19[b]	2.58
6 h	13.17[b]	10.37[b]	2.10
Post-transport			
2 h	4.53[a]	3.23[a]	0.87
4 h	3.29[a]	2.56[a]	0.72

[a,b] Means within a column without a common superscript differ (P < 0.01)

Dehydroepiandrosterone. Transport in individual stalls or in a loose group did not have an effect on dehydroepiandrosterone (DHEA) concentrations in Trials 1 and 2 (Table 3). There was no significant interaction between treatment and sample time (P = 0. 539) and there were no statistically significant differences in DHEA concentrations between treatments (P = 0. 416). Sample-time comparisons also yielded no significant differences.

Table 3. Least squares means of DHEA concentrations (pg/ml) in relation to time relative to start of transport for both treatment groups during Trials 1 and 2.

	Treatment		
Time	Stall	Group	SEM
Pre-transport	499.8	374.4	88.2
Transport			
2 h	672.9	516.6	139.9
4 h	657.9	565.1	143.2
6 h	578.6	753.3	166.2
Post-transport			
2 h	471.3	360.0	90.7
4 h	589.7	339.1	114.8

Hormone Concentrations During Transport. The plasma concentrations of cortisol, corticosterone and DHEA from Trials 1 and 2 were subsequently analyzed at the three sample times during transport only. Again, there were no statistically significant differences found between horses transported in individual stalls versus those transported in a loose group when comparing cortisol ($P = 0.712$), corticosterone ($P = 0.451$) or DHEA ($P = 0.880$). There were also no significant interactions between treatments and sample time.

Trial 3

Transport in individual stalls or in a loose group did not have an effect on plasma concentrations of cortisol, corticosterone and DHEA during Trial 3, the follow-up experiment (Tables 4, 5 and 6). There were no significant interactions between treatment and sample time for cortisol ($P = 0.308$), corticosterone ($P = 0.299$), or DHEA

(P = 0.130). There were also no significant differences between treatment groups in cortisol (P = 0.269), corticosterone (P = 0.681) or DHEA (P = 0.141) concentrations.

Table 4. Least squares means concentrations of cortisol (ng/ml) (± SEM) in relation to time relative to start of transport for both treatment groups during Trial 3.

	Treatment	
Time	Stall	Group
Pre-transport	$9.13^{a} \pm 1.68$	$6.55^{a} \pm 1.67$
Transport		
2 h	$24.73^{b} \pm 2.64$	$23.53^{b} \pm 2.63$
4 h	$27.75^{b} \pm 2.95$	$24.09^{b} \pm 2.94$
6 h	$24.33^{b} \pm 3.24$	$17.71^{c} \pm 3.23$
Post-transport		
2 h	$7.24^{a} \pm 1.42$	$6.71^{a} \pm 1.40$
4 h	$7.17^{a} \pm 1.12$	$6.07^{a} \pm 1.10$

[a,b] Means within a column without a common superscript differ (P < 0.01)

Table 5. Least squares means concentrations of corticosterone (ng/ml) (± SEM) in relation to time relative to start of transport for both treatment groups during Trial 3.

	Treatment	
Time	Stall	Group
Pre-transport	$9.99^{ac} \pm 3.34$	$5.33^{a} \pm 3.33$
Transport		
2 h	$15.14^{b} \pm 2.84$	$11.71^{b} \pm 2.83$
4 h	$16.04^{b} \pm 4.26$	$13.06^{b} \pm 4.25$
6 h	$15.50^{ab} \pm 4.09$	$11.04^{ab} \pm 4.07$
Post-transport		
2 h	$9.68^{abc} \pm 2.56$	$6.05^{a} \pm 2.54$
4 h	$7.25^{c} \pm 3.54$	$11.24^{ab} \pm 3.53$

^{a,b,c} Means within a column without a common superscript differ ($P < 0.05$)

Table 6. Least squares means concentrations of DHEA (pg/ml) (± SEM) in relation to time relative to start of transport for both treatment groups during Trial 3.

	Treatment	
Time	Stall	Group
Pre-transport	$2172^{a} \pm 379$	1006 ± 379
Transport		
2 h	$725^{b} \pm 178$	719 ± 177
4 h	$953^{b} \pm 282$	684 ± 281
6 h	$1141^{b} \pm 162$	798 ± 160
Post-transport		
2 h	$997^{b} \pm 265$	1074 ± 264
4 h	$888^{b} \pm 231$	912 ± 230

^{a,b} Means within a column without a common superscript differ ($P < 0.025$)

Pre-transport Hormone Concentrations

Comparisons of pre-transport concentrations of cortisol, corticosterone and DHEA between Trials 1, 2 and 3 were analyzed to determine effects of change from group paddock housing to individual stall housing (Table 7). Plasma concentrations of each hormone prior to transport were not different between Trials 1 and 2 for cortisol, corticosterone or DHEA ($P = 0.835$, $P = 0.424$ and $P = 0.401$). However, pre-transport concentrations of cortisol between Trials 1 and 3, and between Trials 2 and 3 were significantly different ($P < 0.001$). Similarly, DHEA concentrations in pre-transport samples were significantly different between Trials 1 and 3, as well as between Trials 2 and 3 ($P = 0.001$). There were no significant differences between pre-transport concentrations of corticosterone prior to transport in any of the trials.

Table 7. Mean pre-transport concentrations (± SEM) of each hormone in relation to trial.

	Trial 1	Trial 2	Trial 3
Cortisol (ng/ml)	$2.71^{a} \pm 0.91$	$2.84^{a} \pm 0.84$	$7.87^{b} \pm 1.11$
Corticosterone (ng/ml)	3.76 ± 1.19	4.87 ± 1.14	7.50 ± 2.27
DHEA (pg/ml)	$482^{a} \pm 101$	$392^{a} \pm 73$	$1607^{b} \pm 286$

[a,b]Means within a row without a common superscript differ ($P < 0.01$)

Hormone Comparisons by Diet

Trials 1 and 2. Cortisol, corticosterone and DHEA plasma concentrations from Trials 1 and 2 were analyzed to determine effects of dietary supplement (Table 8). There were no significant differences in cortisol ($P = 0.943$), corticosterone ($P = 0.720$)

or DHEA (P = 0.167) concentrations between horses that were fed no supplement, those that were fed a proteinated form of supplement and those that were fed a sulfated form of supplement.

Table 8. Least squares means of cortisol, corticosterone and DHEA concentrations (± SEM) by dietary supplement treatment during Trials 1 and 2.

	Control	Proteinated	Sulfated
Cortisol (ng/ml)	10.55 ± 2.11	10.52 ± 2.86	11.09 ± 1.83
Corticosterone (ng/ml)	8.74 ± 1.45	10.01 ± 1.89	8.45 ± 1.29
DHEA (pg/ml)	390 ± 124	533 ± 184	640 ± 100

During Transport. Plasma hormone concentrations were reanalyzed for Trials 1 and 2 to include only the transport period (Table 9). The plasma concentrations of corticosterone and DHEA still did not differ significantly between horses fed no dietary supplement, horses fed a proteinated form of supplement and horses fed a sulfated form of supplement. However, the plasma cortisol concentrations of the horses fed a sulfated form of supplement were significantly higher than the cortisol concentrations of the horses fed no dietary supplement and those that were fed a proteinated form of supplement (P = 0.04).

Table 9. Least squares means of cortisol, corticosterone and DHEA concentrations (± SEM) by dietary supplement treatment during the 6 hour transport period of Trials 1 and 2.

	Control	Proteinated	Sulfated
Cortisol (ng/ml)	$16.24^{a} \pm 4.45$	$9.81^{a} \pm 6.65$	$25.52^{b} \pm 3.58$
Corticosterone (ng/ml)	6.47 ± 3.71	19.29 ± 5.60	15.92 ± 2.95
DHEA (pg/ml)	338 ± 229	836 ± 352	631 ± 179

[a,b]Means within a row that lack a common superscript differ ($P < 0.01$)

Trial 3. Cortisol, corticosterone and DHEA plasma concentrations from Trial 3 were analyzed to determine effects of dietary supplement (Table 10). Differences in cortisol and corticosterone concentrations between the groups of horses fed no dietary supplement, a proteinated form of supplement or a sulfated form of supplement were not statistically significant ($P = 0.633$ and $P = 0.215$). However, DHEA concentrations for horses that were fed a sulfated form of supplement in Trial 3 were significantly higher than DHEA concentrations of horses fed a proteinated form of the supplement ($P = 0.002$).

Table 10. Least squares means of cortisol, corticosterone and DHEA concentrations (± SEM) for dietary supplement treatment groups in Trial 3, the follow-up experiment.

	Control	Proteinated	Sulfated
Cortisol (ng/ml)	16.43 ± 1.65	14.88 ± 1.74	14.95 ± 1.65
Corticosterone (ng/ml)	9.62 ± 2.57	14.17 ± 2.72	9.22 ± 2.57
DHEA (pg/ml)	$857^{ab} \pm 170$	$649^{a} \pm 181$	$1511^{b} \pm 170$

[a,b]Means within a row that lack a common superscript differ ($P < 0.001$)

RNA Analysis

Analysis of the four RNA samples on the plate coated with primers for human inflammatory response genes yielded quality amplification of 13 out of 84 of the target genes (Appendix Table 12). From the 13 target genes that amplified, 12 of them showed differing expression among the 4 samples (Fig. 6) as measured by the calculation of the cycle at which the amplification curve crossed the threshold. The 1 remaining gene showed strong amplification, but did not differ in the threshold cycle (C_t) value between samples. Five additional primer sets were included on the plate to be used as housekeeping genes. The primers selected for housekeeping genes typically show minimal differences in C_t values for all samples indicating little or no variation based on inflammatory response for the target species, thus making them useful for data correction. Of the 5 of the housekeeping genes included on the plate used, the samples amplified on only one primer set (Appendix Table 12). Results from the genomic contamination wells, reverse transcription control wells and positive PCR controls indicated that the samples performed well with no contamination.

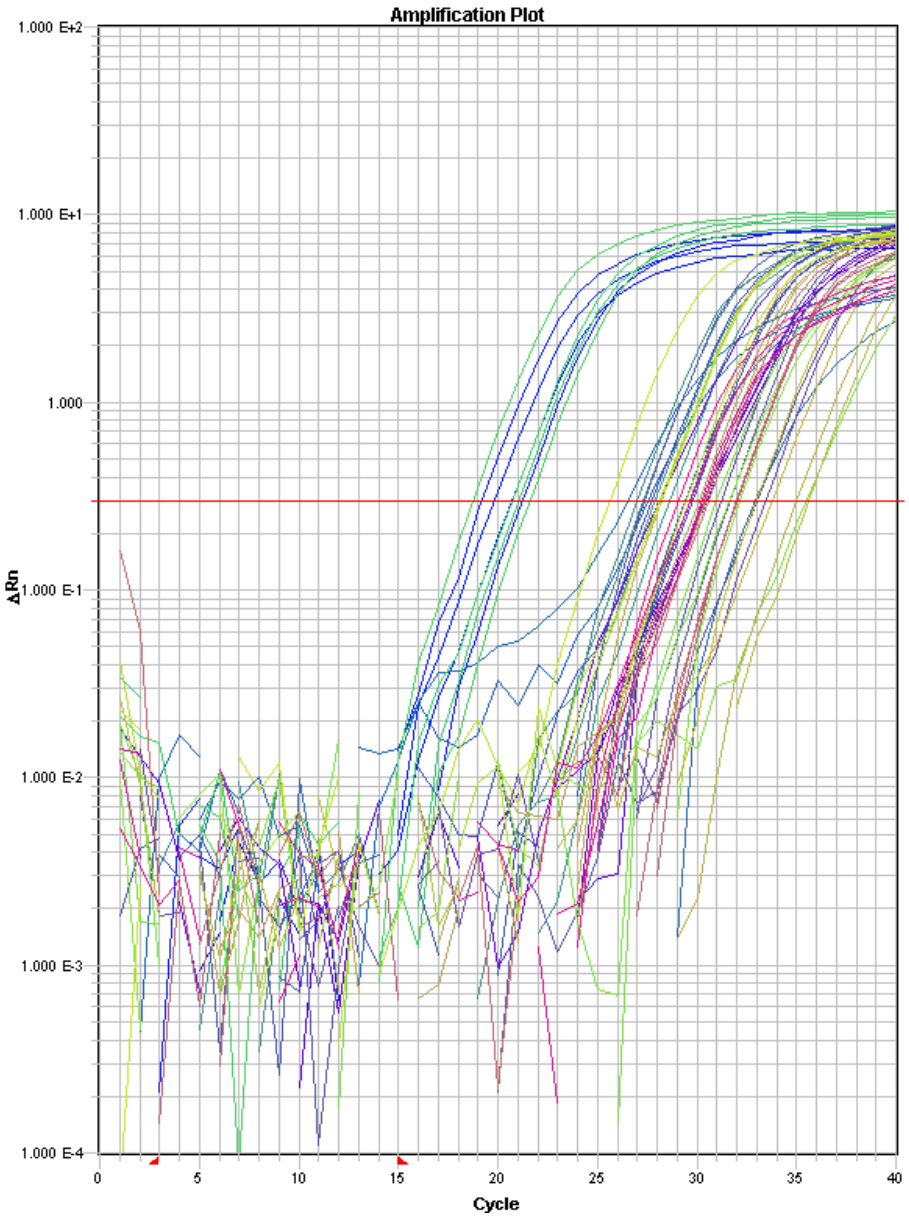

Figure 6. Plot of amplification of four samples chosen for preliminary analysis of inflammatory gene expression using human primers. Differing colors represent individual genes and each line represents an individual sample. Space between like colored lines indicated differences in gene expression. All genes that failed to amplify are absent for clarity. The red line indicates threshold.

DISCUSSION

Results from this study indicate that transporting horses in individual stalls or in loose groups does not reduce the level of stress experienced by naïve horses during transport. These findings are inconsistent with a 2002 study conducted at the University of California at Davis, which concluded that horses transported for 24 hours in loose groups had decreased physiological measures of stress when compared to horses that were individually stalled (Stull and Rodiek, 2002). That study consisted of 10 mature horses being transported for 24 hours in a switchback design where all horses received both treatments. All horses had prior transport experience, and any potential confounding effects of that prior experience were not discussed in the article. The researchers collected blood samples at 0, 3, 12 and 24 hours of transport and evaluated a multitude of physiological parameters including packed cell volume, serum total protein, lactate and creatine kinase concentrations, cortisol and glucose concentrations, white blood cell counts, neutrophil:lymphocyte ratios, aminotransferase and alpha one acidglycoprotein concentrations. While the results concur with our observations of increased cortisol concentrations during transport for all horses, it is unclear if the researchers found significant differences between cortisol concentrations of horses in different treatment groups during transport. From the information presented, it would appear that our observations of similar cortisol concentrations for both treatment groups after 2 h and 4 h of transport are consistent with the findings of Stull and Rodiek (2002) up to 3 h of transport. However, Stull and Rodiek reported a divergence of cortisol

concentrations after 3 h of transport, with cross-tied horses expressing higher concentrations and loose horses expressing lower concentrations. Over the 24 h transport period that Stull and Rodiek studied, they reported significant differences between treatment groups in white blood cell counts, glucose and cortisol concentrations and neutrophil:lymphocyte ratios at a significance level of $P < 0.05$ (Stull and Rodiek, 2002). It was unclear from the information presented what the significance of each parameter was, and at which sample times the differences were significant. It was also unclear if any treatment by sample time interactions were found. While the researchers reported differences in cortisol concentrations between treatment groups over a 24 h period of transport, results from this study found no differences between the treatment groups between 3 h and 6 h.

When this study was designed, the horses that were assigned to each treatment were chosen by blocking for supplement treatment and temperament. This caused horses that were not being housed together to be transported together in a loose group. When the horses were loaded into the trailer, the loose group spent several minutes biting, kicking and vocalizing to establishment of a dominance structure. It is unknown if the group horses continued to display aggressive behavior during the time they were being transported. The effect that social dominance had on glucocorticoid and DHEA concentrations within the loose group of horses is unknown.

Horses that are transported in high density versus low density groups are more likely to fall or sustain injuries during transport (Collins et al., 2000). In this study, the horses were carefully monitored during and after the transport period for falls or injuries

sustained during transport. A brief visual inspection of the horses in the trailer was performed every hour during transport. There was only one fall and 3 minor injuries that occurred over the course of the study. All of those animals were in the group compartment at the time of the fall or injury, but all occurred on different days of transport. In the case of the animal that fell, a visual inspection was made by the driver after 5 h of transport and all horses were upright in the trailer. After 6 h of transport, an individual in the group compartment was noticed to be on the floor of the trailer in an upright recumbent position with its head up and alert, and legs curled under the body. The other horses in the group were removed, but the horse did not attempt to climb to its feet. A researcher tugged the halter of the animal to encourage it to rise, but again, the horse showed no attempt. Only after a second researcher began pushing the horse in the hind quarters at the same time the first researcher tugged at the halter, did the horse rise to its feet. The horse appeared to exhibit what Overmier and Seligman defined as Learned Helplessness; where an animal that is presented with an adverse stimulus with no ability to stop it, will cease its attempts to avoid the stimulus (Overmier and Seligman, 1967). The animal's apathy toward standing when given space and opportunity, strongly indicated to this researcher that it stopped attempting to escape the fallen position prior to our presence. Scrapes on the back and legs of the horse suggest that the animal had been stepped on by other animals in the group during the time that it was down, however, it sustained no major injuries and unloaded without hesitation or haste. The 6 h transport blood sample was taken immediately after the horse was brought to its feet, and glucocorticoid and DHEA concentrations were closely examined

for this individual animal for any variation that may be due to the fall. All hormone concentrations decreased from the previous sample at a rate consistent with other animals being transported at that time. No indication that the fall caused significant changes in the horse's hormone concentrations was noted, so data collected from the fallen horse was analyzed in the statistical model.

While glucocorticoids and DHEA concentrations were measured up to 4 hours post-transport and returned to pre-transport concentrations quickly, researchers monitoring the horses feed consumption indicated a possibility of residual effects of the transport in the form of reduced feed intake (Table 11). A tendency for reduced intake was noted in Trial 1 were the average amount of hay refused during the 5 d period after transport was 1.7 times higher than the 5 d period prior to transport. Differences in hay refusal amounts in Trials 2 and 3 were equal and negligible, possibly indicating an acclimation to transport after the first trial.

Table 11. Average amounts of hay refused in time relative to transport for each trial.

Time Period	Hay Refused (kg)
Trial 1	
5 days pre-transport	0.191
5 days post-transport	0.325
Trial 2	
5 days pre-transport	0.257
5 days post-transport	0.299
Trial 3	
5 days pre-transport	0.48
5 days post-transport	0.413

It should be noted that the horses exercise programs were not consistent prior to transport in Trial 3. All horses were being exercised, however, half of them were being lounged for 15 min per day, then handled and groomed for an additional 45 min per day, 5 days per week, while the other half were lounged for 15 min per day, 3 days per week and turned out in groups of 2 for 45 min per day, 2 days per week. After 15 days, the exercise assignments were reversed, such that every horse received equal exercise treatments over the inter-trial period. This difference in exercise programs at the time of the transport trial could have an effect on glucocorticoid and DHEA concentrations found in samples taken during Trial 3.

Differing exercise programs or individual stall housing may have contributed to an anomaly observed in the overall DHEA pattern in Trial 3. Concentrations of DHEA did not fluctuate significantly during Trials 1 and 2, however, in Trial 3, pre-transport concentrations of DHEA were significantly higher than the previous 2 trials, then DHEA decreased significantly during transport. While we observed a pattern of increased concentrations of cortisol and corticosterone during transport in all three trials, a decrease in DHEA during transport was not observed in Trials 1 and 2. Because of multiple changes to the horses' environment and routine, and a lack of replication of the follow-up experiment, it is unclear at this time what may have caused this spike pre-transport and subsequent reduction in transport DHEA concentrations.

When comparing hormone concentrations between the first two trials and Trial 3, the blood samples collected during Trial 3 were not passed through the leukocyte filtration system prior to centrifugation, as happened in Trials 1 and 2. Technical

representatives at Ambion, the manufacturer of the filtration system, noted that there should be no effect on the hormone concentrations within the plasma as a result of the filtration process. The pore size of the filter is quite large in an effort to only capture white blood cells. We did not, however, compare any samples by filtering a portion of the sample and testing both for accuracy. Although it is reasonable to assume no effect, a comparison between filtered and non-filtered blood could be useful.

CONCLUSIONS

Comparisons of plasma hormone concentrations between treatment groups in Trials 1 and 2 indicated that being transported in an individual stall versus a loose group did not have a significant effect on the amount of glucocorticoids and DHEA that are expressed in response to stress experienced during transport. Hormone data from the follow-up experiment further supports the conclusion that there are no notable differences between concentrations of horses individually stalled during transport versus those transported in a loose group.

The concentrations of cortisol and corticosterone observed for each animal during each of the three trials in this study clearly indicated that transport caused a significant rise in glucocorticoids, which subsequently returned to pre-transport concentrations within 2 hours of unloading. This suggests that transport is a stressor for naïve horses, and that resistance of the stressor through glucocorticoid expression subsides quickly after the termination of the stressor.

Horses fed different dietary supplements showed no significant differences in hormone concentrations in the absence of a stressor. However, during the transport period in the first two trials, horses that were fed a sulfated form of supplement exhibited significantly higher concentrations of cortisol. In addition, horses fed the sulfated form of supplement expressed significantly higher concentrations of DHEA in the follow-up experiment than horses that were fed a proteinated form of supplement. This suggests

that horses fed a sulfated form of the mineral supplement may increase the hormone response to a stressor.

The comparison of pre-transport cortisol and DHEA concentrations from the first two trials to Trial 3 suggest that maintaining the horses in individual stalls versus group paddocks resulted in increased basal concentrations of each of these hormones, and may suggest that additional underlying stress was experienced by the horses.

Preliminary data obtained from the amplification of RNA samples suggest that some primers for inflammation related genes in humans may be useful in the detection of stress related physiological reactions in horses. Further investigation is needed to determine if analysis of inflammatory-related gene expression by real-time PCR may provide a reliable index of stress in horses and other species.

LITERATURE CITED

American Horse Council. 2005. The economic impact of the horse industry on the United States. American Horse Council Foundation, Washington, DC.

Agriculture and Resource Management Council of Australia and New Zealand (ARMCANZ). 1987. Model Code of Practice for the welfare of animals: Land Transport of Horses. CSIRO Publishing, Collingswood, Victoria.

Collins, M. N., T. H. Friend, F. D. Jousan and S.C. Chen. 2000. Effects of density on displacement, falls, injuries, and orientation during horse transportation. Appl. Anim. Behav. Sci. 67:169-179.

Council of the European Union. 2005. Council Regulation No. 1/2005: On the protection of animals during transport and related operations. Official Journal of the European Union. Luxembourg.

Clark, D. K., T. H. Friend and G. Dellmeier. 1993. The effect of orientation during trailer transport on heart rate, cortisol and balance in horses. Appl. Anim. Behav. Sci. 38:179-189.

Davidson, N. and P. Harris. 2002. Nutrition and Welfare. Pages 45 – 76 in The Welfare of Horses. N. Waran, ed. Kluwer Academic Publishers, Norwell, MA.

Eldridge, G. A., C. G. Winfield and D. J. Cahill. 1988. Responses of cattle to different space allowances, pen sizes and road conditions during transport. Aust. J. Exp. Agr. 28:155-159.

Friend, T. H. 2000. Dehydration, stress, and water consumption of horses during long-distance commercial transport. J. Anim. Sci. 78:2568-2580.

Friend, T. H., M. T. Martin, D. D. Householder and D. M. Bushong. 1998. Stress responses of horses during a long period of transport in a commercial truck. J. Am. Vet. Med. Assoc. 212:838-844.

Gibbs, A. E. and T. H. Friend. 1999. Horse preference for orientation during transport and the effect of orientation on balancing ability. Appl. Anim. Behav. Sci. 63:1-9.

Giovagnoli, G., M. Trabalza Marinucci, A. Bolla and A. Borghese. 2002. Transport stress in horses: An electromyographic study on balance preservation. Livest. Prod. Sci. 73:247-254.

Goodwin, D. 2002. Horse Behaviour: Evolution, Domestication and Feralisation. Pages 1 – 18 in The Welfare of Horses. N. Waran, ed. Kluwer Academic Publishers, Norwell, MA.

Heuser, I., M. Deuschle, P. Luppa, U. Schweiger, H. Standhardt and B. Weber. 1998. Increased diurnal plasma concentrations of dehydroepiandrosterone in depressed patients. J. Clin. Endocr. Metab. 83:3130-3133.

Houpt, K. A. 1991. Domestic Animal Behavior for Veterinarians and Animal Scientists. Iowa State University Press, Ames, IA.

Iacono, C., T. H. Friend, H. Keen, T. Martin and P. Krawczel. 2007. Effects of density and water availability on the behavior, physiology, and weight loss of slaughter horses during transport. J. Equine Vet. Sci. 27:355-361.

Illinois General Assembly. 2007. Public Act 095-0002. House Bill 1711: An amendment to the Illinois Horse Meat Act. Illinois General Assembly, Springfield, IL.

Kusunose, R. and K. Torikai. 1996. Behavior of untethered horses during vehicle transport. J. Equine Sci. 7:21-26.

Monague, K. R., A. I. Leshner and D. K. Candland. 1975. Dominance status and adrenocortical reactivity to stress in squirrel monkeys (Saimiri sciureus). Primates 16:457-463.

Montana Legislature. 2009. Sixty-first session. House Bill No. 418. An Act authorizing investor-owned equine slaughter or processing facilities; prohibiting a court from granting an injunction to stop or delay the construction of an equine slaughter or processing facility based on legal challenges or appeals of a permit, license, certificate, or other approval issued in conjunction with environmental laws; and setting bonding requirements. Montana Legislature, Helena, MT.

Nolen, R. S. 2008. U.S. horse slaughter exports to Mexico increase 312%. J. Am. Vet. Med. Assoc. News Jan. 15, 2008. http://www.avma.org/onlnews/javma/jan08/080115a.asp Accessed Feb. 26, 2009.

North Dakota Legislative Assembly. 2009. Sixty-first session. Reengrossed House Bill No. 1496. An Act to provide for an equine processing facility feasibility study; and to provide an appropriation. North Dakota Legislative Assembly, Bismarck, ND.

Oikawa, M., S. Hobo, T. Oyamada and H. Yoshikawa. 2005. Effects of orientation, intermittent rest and vehicle cleaning during transport on development of transport-related respiratory disease in horses. J. Comp. Pathol. 132:153-168.

Oikawa, M., S. Takagi, R. Anzai, H. Yoshikawa and T. Yoshikawa. 1995. Pathology of equine respiratory disease occurring in association with transport. J. Comp. Pathol. 113:29-43.

Opstad, P. K. 1992. Androgenic hormones during prolonged physical stress, sleep, and energy deficiency. J. Clin. Endocr. Metab. 74:1176-1183.

Overmier, J. B. and M. E. P. Seligman. 1967. Effects of inescapable shock upon subsequent escape and avoidance responding. J. Comp. Physiol. Psych. 63:28-33.

Parker, R., R. Watson, E. Wells, S. N. Brown, C. J. Nicol and T. G. Knowles. 2004. The effect of blindfolding horses on heart rate and behaviour during handling and loading onto transport vehicles. Anim. Welfare 13:433-437.

Parkinson, L., E. Ghyoot, R. Van Vlierberghe and G. Bone. 1960. Adrenocortical response of Bantus and Europeans to surgical stress. T. Roy. Soc. Trop. Med. H. 54:366-372.

Schedlowski, M., D. Wiechert, T. O. F. Wagner and U. Tewes. 1992. Acute psychological stress increases plasma levels of cortisol, prolactin and TSH. Life Sci. 50:1201-1205.

Scott, L. V., F. Svec and T. Dinan. 2000. A preliminary study of dehydroepiandrosterone response to low-dose ACTH in chronic fatigue syndrome and in healthy subjects. Psychiat. Res. 97:21-28.

Selye, H. 1951. The General Adaptation Syndrome and the Diseases of Adaptation. Am. J. Med. 10:549-555.

Smith, B. L., J. H. Jones, W. J. Hornof, J. A. Miles, K. E. Longworth and N. H. Willits. 1996. Effects of road transport on indices of stress in horses. Equine Vet. J. 28:446-454.

South Dakota Legislative Assembly. 2009. Eighty-fourth session. Senate Bill No. 114. An Act to provide for a study of the feasibility of establishing an equine processing facility and to make an appropriation therefor. South Dakota Legislative Assembly, Pierre, SD.

Stull, C. L. 1999. Responses of horses to trailer design, duration and floor area during commercial transportation to slaughter. J. Anim. Sci. 77:2925-2933.

Stull, C. L. and A. V. Rodiek. 2000. Physiological responses of horses to 24 hours of transportation using a commercial van during summer conditions. J. Anim. Sci. 78:1458-1466.

Stull, C. L. and A. V. Rodiek. 2002. Effects of cross-tying horses during 24 h of road transport. Equine Vet. J. 34:550-555.

Tarrant, P. V., F. J. Kenny, D. Harrington and M. Murphy. 1992. Long distance transportation of steers to slaughter: Effect of stocking density on physiology, behaviour and carcass quality. Livest. Prod. Sci. 30:223-238.

Texas Statutes Agriculture Code. 1949. Title 6: Production, processing and sale of animal products. Chapter 149: Sale of Horse Meat for Human Consumption. Texas Legislature, Austin, TX.

Toscano, M. J. and T. H. Friend. 2001. A note on the effects of forward and rear-facing orientations on movement of horses during transport. Appl. Anim. Behav. Sci. 73:281-287.

U.S. CFR. 2002. Title 9: Animals and animal products. Chapter 88: Commercial Transportation of Equines for Slaughter. U.S. Government Printing Office, Washington, DC.

USDA NASS. 2007a. Census of Agriculture: Table 31. Other Animals and Animal Products – Inventory and Number Sold: 2007 and 2002. U.S. Department of Agriculture, Washington, DC.

USDA NASS. 2007b. Quick Statistics: United States Slaughter Weekly Report by Species and Class: Equine. 1980-2007. http://www.nass.usda.gov/Data_and_Statistics/Quick_Stats/ Accessed Feb 26, 2009.

U.S. House of Representatives. 2002. 107th Congress. H.R. 3781: American Horse Slaughter Prevention Act. U.S. Congress, Washington, DC.

U.S. House of Representatives. 2003. 108th Congress. H.R. 857: American Horse Slaughter Prevention Act. U.S. Congress, Washington, DC.

U.S. House of Representatives. 2005. 109th Congress. H.R. 503: Horse Slaughter Prohibition bill. U.S. Congress, Washington, DC.

U.S. House of Representatives. 2007. 110th Congress. H.R. 503: To amend the Horse Protection Act to prohibit the shipping, transporting, moving, delivering, receiving, possessing, purchasing, selling or donation of horses and other equines to be slaughtered for human consumption, and for other purposes. U.S. Congress, Washington, DC.

U.S. House of Representatives. 2008. 110th Congress. H.R. 6598: Prevention of Equine Cruelty Act of 2008. U.S. Congress, Washington, DC.

U.S. House of Representatives. 2009a. 111th Congress. H.R. 305: Horse Transportation Safety Act of 2009. U.S. Congress, Washington, DC.

U.S. House of Representatives. 2009b. 111th Congress. H.R. 503: Prevention of Equine Cruelty Act of 2009. U.S. Congress, Washington, DC.

U.S. Senate. 2004. 108th Congress. S. 2352: American Horse Slaughter Prevention Act of 2004. U.S. Congress, Washington, DC.

U.S. Senate. 2005. 109th Congress. S. 1915: Virgie S. Arden American Horse Slaughter Prevention Act. U.S. Congress, Washington, DC.

Waran, N. K., V. Robertson, D. Cuddeford, A. Kokoszko and D. J. Marlin. 1996. Effects of transporting horses facing either forwards or backwards on their behaviour and heart rate. Vet. Rec.139:7-11.

Waring, G. H. 1983. Horse Behavior: The Behavioral Traits and Adaptations of Domestic and Wild Horses, Including Ponies. Noyes Publications, Park Ridge, NJ.

Whiting, T. 1999. Maximum loading density of loose horses. Can. J. Anim. Sci. 79:115-118.

Yehuda, R., S. R. Brand, J. A. Golier and R-K. Yang. 2006. Clinical correlates of DHEA associated with port-traumatic stress disorder. Acta. Psychiat. Scand. 114:187-193.

APPENDIX

Table 12. PCR evaluation of gene expression using primers for target inflammation genes in humans.

Result	Gene ID	Gene Description
-	ABCF1	ATP-binding cassette, sub-family F (GCN20), member 1
**	BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51)
-	C3	Complement component 3
-	C4A	Complement component 4A (Rodgers blood group)
-	C5	Complement component 5
-	CCL1	Chemokine (C-C motif) ligand 1
-	CCL11	Chemokine (C-C motif) ligand 11
-	CCL13	Chemokine (C-C motif) ligand 13
-	CCL15	Chemokine (C-C motif) ligand 15
-	CCL16	Chemokine (C-C motif) ligand 16
-	CCL17	Chemokine (C-C motif) ligand 17
-	CCL18	Chemokine (C-C motif) ligand 18
-	CCL19	Chemokine (C-C motif) ligand 19
-	CCL2	Chemokine (C-C motif) ligand 2
-	CCL20	Chemokine (C-C motif) ligand 20
-	CCL21	Chemokine (C-C motif) ligand 21
-	CCL23	Chemokine (C-C motif) ligand 23
**	CCL24	Chemokine (C-C motif) ligand 24
-	CCL25	Chemokine (C-C motif) ligand 25
-	CCL26	Chemokine (C-C motif) ligand 26
-	CCL3	Chemokine (C-C motif) ligand 3
-	CCL4	Chemokine (C-C motif) ligand 4
-	CCL5	Chemokine (C-C motif) ligand 5
-	CCL7	Chemokine (C-C motif) ligand 7
-	CCL8	Chemokine (C-C motif) ligand 8
-	CCR1	Chemokine (C-C motif) receptor 1
-	CCR2	Chemokine (C-C motif) receptor 2
-	CCR3	Chemokine (C-C motif) receptor 3
**	CCR4	Chemokine (C-C motif) receptor 4
-	CCR5	Chemokine (C-C motif) receptor 5
-	CCR6	Chemokine (C-C motif) receptor 6
**	CCR7	Chemokine (C-C motif) receptor 7
-	CCR8	Chemokine (C-C motif) receptor 8
*	CCR9	Chemokine (C-C motif) receptor 9
**	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta

***** Denotes genes that exhibited amplification with no expression differences between samples

****** Denotes genes that exhibited amplification and showed differences in expression between samples

Table 12. Continued

Result	Gene ID	Gene Description
-	CRP	C-reactive protein, pentraxin-related
**	CX3CR1	Chemokine (C-X3-C motif) receptor 1
-	CXCL1	Chemokine (C-X-C motif) ligand 1
-	CXCL10	Chemokine (C-X-C motif) ligand 10
-	CXCL11	Chemokine (C-X-C motif) ligand 11
**	CXCL12	Chemokine (C-X-C motif) ligand 12
-	CXCL13	Chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
-	CXCL14	Chemokine (C-X-C motif) ligand 14
-	CXCL2	Chemokine (C-X-C motif) ligand 2
-	CXCL3	Chemokine (C-X-C motif) ligand 3
-	CXCL5	Chemokine (C-X-C motif) ligand 5
-	CXCL6	Chemokine (C-X-C motif) ligand 6
-	CXCL9	Chemokine (C-X-C motif) ligand 9
-	ICEBERG	ICEBERG caspase-1 inhibitor
-	IFNA2	Interferon, alpha 2
-	IL10	Interleukin 10
-	IL10RA	Interleukin 10 receptor, alpha
-	IL10RB	Interleukin 10 receptor, beta
-	IL13	Interleukin 13
-	IL13RA1	Interleukin 13 receptor, alpha 1
-	IL17C	Interleukin 17C
-	IL1A	Interleukin 1, alpha
-	IL1B	Interleukin 1, beta
-	IL1F10	Interleukin 1 family, member 10 (theta)
**	IL1F5	Interleukin 1 family, member 5 (delta)
-	IL1F6	Interleukin 1 family, member 6 (epsilon)
-	IL1F7	Interleukin 1 family, member 7 (zeta)
-	IL1F8	Interleukin 1 family, member 8 (eta)
-	IL1F9	Interleukin 1 family, member 9
-	IL1R1	Interleukin 1 receptor, type I
-	IL1RN	Interleukin 1 receptor antagonist
-	IL22	Interleukin 22
-	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)
-	IL5RA	Interleukin 5 receptor, alpha
-	IL8	Interleukin 8
-	IL8RA	Interleukin 8 receptor, alpha
-	IL8RB	Interleukin 8 receptor, beta
-	IL9	Interleukin 9

** Denotes genes that exhibited amplification and showed differences in expression between samples

Table 12. Continued

Result	Gene ID	Gene Description
-	IL9R	Interleukin 9 receptor
-	LTA	Lymphotoxin alpha (TNF superfamily, member 1)
-	LTB	Lymphotoxin beta (TNF superfamily, member 3)
-	LTB4R	Leukotriene B4 receptor
**	MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)
-	SCYE1	Small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)
-	SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
**	TNF	Tumor necrosis factor (TNF superfamily, member 2)
**	CD40LG	CD40 ligand (TNF superfamily, member 5, hyper-IgM syndrome)
-	TOLLIP	Toll interacting protein
**	XCR1	Chemokine (C motif) receptor 1
-	B2M	Beta-2-microglobulin
**	HPRT1	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)
-	RPL13A	Ribosomal protein L13a
-	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
-	ACTB	Actin, beta
**	HGDC	Human Genomic DNA Contamination
**	RTC	Reverse Transcription Control
**	RTC	Reverse Transcription Control
**	RTC	Reverse Transcription Control
**	PPC	Positive PCR Control
**	PPC	Positive PCR Control
**	PPC	Positive PCR Control

** Denotes genes that exhibited amplification and showed differences in expression between samples

Table 13. Gene expression as quantified by threshold cycle values for each amplified gene for a low cortisol responding animal (72) and a high cortisol responding animal (705). Refer to Table 12 for descriptions of each gene.

	Sample Identification			
	72		705	
Gene ID	Pre-transport	4 h	Pre-transport	4 h
BCL6	20.73	19.85	21.26	19.27
CCL24	27.53	26.52	29.33	32.91
CCR4	27.8	29.77	29.65	27.44
CCR7	27.06	28.67	27.38	27.94
CCR9	27.14	27.54	27.36	27.77
CEBPB	21.07	20.74	21.64	18.79
CX3CR1	30.39	30.42	30.53	28.18
CXCl12	31.31	33.06	33.5	31.72
IL1F5	31.01	31.66	35.56	32.13
MIF	32.07	32	32.07	30.65
TNF	30.18	30.49	29.69	28.27
CD40LG	35.09	35.66	33.81	33
XCR1	28.21	28.28	29.5	25.66
HPRT1	30.18	30.32	29.64	29.01

Table 14. Cortisol concentrations (ng/ml) of stalled horses in relation to time relative to start of transport in Trials 1 and 2.

Animal	Pre-transport	Transport			Post-transport	
ID		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
53	0.77	8.76	9.19	10.65	0.86	0.71
57	1.31	9.85	10.58	9.72	3.63	3.67
58	1.24	17.27	11.83	9.87	1.58	1.25
63	0.75	7.23	12.05	10.30	1.03	0.66
66	0.66	11.76	17.56	9.01	4.67	1.91
72	2.20	9.19	13.82	10.18	5.25	7.83
76	1.15	6.76	7.56	9.21	0.90	0.80
205	2.08	17.41	8.71	7.88	2.63	2.65
209	0.98	9.66	7.93	5.72	5.14	3.76
310	1.40	31.59	35.98	36.74	5.46	1.44
612	16.30	43.25	38.49	29.92	11.36	11.39
614	1.31	16.46	17.90	10.58	2.03	3.89
615	1.10	9.34	9.45	6.06	0.65	0.70
618	0.60	15.60	15.60	11.76	0.86	0.76
705	11.80	62.65	83.95	69.17	27.67	19.76
710	0.72	11.45	9.32	6.72	0.85	0.76
711	0.56	12.42	7.79	8.97	0.56	0.65
720	2.31	39.01	41.23	34.29	5.35	4.10
723	3.95	26.91	27.53	17.98	3.91	7.65
726	3.68	19.61	18.50	16.32	1.91	2.98

Table 15. Cortisol concentrations (ng/ml) of group horses in relation to time relative to start of transport in Trials 1 and 2.

Animal	Pre-transport	Transport			Post-transport	
ID		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
53	2.49	11.50	16.04	12.61	1.54	2.57
57	2.78	10.07	13.75	12.16	5.23	5.17
58	1.73	18.28	18.11	16.86	2.20	1.06
63	0.48	10.86	18.25	14.46	0.50	0.67
66	2.66	19.82	13.29	11.27	4.12	4.36
72	0.96	7.08	10.08	8.69	2.60	2.59
76	0.90	4.66	7.42	3.65	0.89	0.78
205	3.21	22.92	19.48	13.15	5.82	5.44
209	0.32	2.64	6.77	4.33	1.12	0.55
310	1.08	12.53	22.26	14.14	1.28	1.34
612	6.97	13.20	18.99	11.88	5.47	3.91
614	0.84	12.88	15.53	8.49	0.66	1.83
615	0.45	8.13	6.43	5.27	0.46	0.42
618	0.61	21.91	13.44	13.30	0.89	0.53
705	15.87	69.31	77.45	60.78	35.82	3.61
710	0.83	11.48	15.19	16.62	0.90	1.03
711	1.04	5.47	2.75	2.12	0.76	0.67
720	3.23	25.33	30.35	25.82	4.30	3.37
723	8.92	29.58	30.05	27.52	8.76	7.51
726	0.94	22.05	20.69	17.00	2.04	1.59

Table 16. Corticosterone concentrations (ng/ml) of stalled horses in relation to time relative to start of transport in Trials 1 and 2.

Animal ID	Pre-transport	Transport			Post-transport	
		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
53	1.12	5.51	6.82	8.30	2.97	0.42
57	0.53	5.09	6.69	7.54	1.77	0.68
58	2.23	7.29	6.40	5.54	2.88	0.55
63	0.27	5.26	8.24	4.47	2.92	0.90
66	1.66	8.56	10.46	7.02	2.85	1.50
72	0.40	5.24	3.99	3.12	0.26	0.44
76	7.69	19.80	19.80	21.42	5.20	2.40
205	19.34	35.02	14.32	22.64	6.82	4.37
209	1.78	13.48	10.98	8.04	2.04	4.96
310	6.36	28.76	27.78	23.17	14.83	18.37
612	24.01	36.67	33.83	29.02	16.68	7.60
614	6.27	48.53	25.19	29.02	8.87	4.67
615	14.94	29.02	13.43	10.92	3.74	3.64
618	1.28	6.29	5.33	4.36	2.28	1.16
705	2.66	12.23	7.75	7.96	1.63	2.19
710	1.37	9.83	7.56	5.99	2.87	1.88
711	6.06	45.58	48.23	38.25	6.32	4.63
720	2.03	12.35	8.39	9.04	1.70	2.03
723	2.13	10.94	7.57	7.77	1.48	1.41
726	3.61	12.81	9.36	9.76	2.55	2.05

Table 17. Corticosterone concentrations (ng/ml) of group horses in relation to time relative to start of transport in Trials 1 and 2.

Animal ID	Pre-transport	Transport			Post-transport	
		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
53	1.05	5.99	6.16	4.42	0.82	3.03
57	0.89	10.80	6.51	6.69	2.91	3.59
58	1.34	7.98	8.03	5.68	0.95	1.83
63	1.56	8.52	8.00	7.53	0.37	0.70
66	1.23	6.38	4.18	3.03	2.02	1.12
72	0.60	3.96	3.54	0.60	0.27	1.11
76	0.72	7.24	13.58	8.80	3.45	3.04
205	0.40	4.44	9.49	6.08	1.07	0.50
209	3.16	9.35	10.30	5.02	2.19	1.62
310	7.75	29.63	33.88	23.90	8.84	6.92
612	9.07	11.33	14.88	14.18	3.26	3.44
614	8.24	43.76	32.51	17.08	3.87	6.68
615	6.58	16.13	14.50	11.19	4.43	6.74
618	0.30	5.56	3.38	3.48	2.29	1.41
705	4.48	7.32	7.18	9.15	1.78	0.43
710	1.83	7.86	9.63	11.82	2.41	1.37
711	6.18	34.63	48.07	38.69	15.48	1.83
720	1.82	8.18	8.15	7.76	1.13	0.90
723	2.81	10.93	9.34	10.93	3.61	1.34
726	6.92	34.84	12.50	11.36	3.58	3.61

Table 18. Dehyrdoepiandrosterone (DHEA) concentrations (pg/ml) of stalled horses in relation to time relative to start of transport in Trials 1 and 2.

Animal	Pre-transport	Transport			Post-transport	
ID		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
53	813	12	1631	1567	828	844
57	102	173	184	105	67	188
58	102	85	34	108	102	127
63	63	1938	2365	113	136	66
66	895	1570	799	534	1981	1595
72	636	581	619	465	526	652
76	146	137	303	278	95	80
205	712	798	412	928	477	532
209	881	1834	1809	1716	184	1015
310	390	344	355	511	283	173
612	145	240	284	1774	185	328
614	133	231	289	175	103	219
615	196	471	335	103	321	272
618	102	158	74	197	29	27
705	898	416	469	361	200	343
710	460	802	89	416	528	498
711	859	861	281	593	962	866
720	262	423	392	217	473	549
723	1557	1736	954	859	1474	2786
726	643	648	1479	551	472	633

Table 19. Dehyrdoepiandrosterone (DHEA) concentrations (pg/ml) of group horses in relation to time relative to start of transport in Trials 1 and 2.

Animal	Pre-transport	Transport			Post-transport	
ID		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
53	54	94	52	105	109	157
57	72	1716	1615	1626	56	144
58	345	120	581	76	267	270
63	168	169	218	337	178	154
66	218	158	2365	2038	111	114
72	274	309	501	295	256	320
76	166	57	54	93	946	573
205	1207	476	714	456	937	1268
209	204	142	163	3770	622	555
310	280	370	259	221	45	270
612	249	134	164	181	166	278
614	55	412	276	425	194	129
615	249	118	124	134	293	239
618	148	43	87	872	842	12
705	976	609	404	648	421	151
710	189	1994	1885	1938	90	149
711	1164	2291	748	811	1105	1478
720	980	352	381	565	62	70
723	385	299	221	252	369	360
726	104	469	489	222	130	90

Table 20. Cortisol concentrations (ng/ml) of stalled horses in relation to time relative to start of transport in Trial 3.

Animal	Pre-transport	Transport			Post-transport	
ID		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
53	10.64	20.35	29.11	24.57	14.11	12.06
63	2.23	18.11	17.85	9.49	1.73	4.73
66	10.32	19.19	13.69	22.14	8.57	9.31
72	8.16	21.85	28.93	14.39	7.59	8.59
310	6.78	30.72	27.30	32.41	7.57	6.61
612	18.88	29.07	33.27	28.31	7.55	8.37
614	5.35	24.17	24.11	17.15	2.52	2.79
705	17.38	46.03	55.51	55.90	9.38	8.29
720	10.46	24.70	32.36	27.64	4.16	5.95
723	2.16	14.19	16.46	12.41	10.35	6.11

Table 21. Cortisol concentrations (ng/ml) of group horses in relation to time relative to start of transport in Trial 3.

Animal	Pre-transport	Transport			Post-transport	
ID		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
57	5.77	18.80	24.00	16.57	6.12	8.90
58	3.98	19.31	28.30	17.49	4.46	5.05
76	4.28	24.03	24.78	22.08	2.95	1.94
205	16.48	37.94	34.54	27.52	18.47	15.12
209	7.25	27.47	20.96	19.33	4.41	4.32
615	2.66	15.66	22.47	15.50	4.65	3.46
618	4.32	26.48	23.57	15.00	5.51	3.39
710	3.60	25.39	17.64	13.74	7.35	3.71
711	9.41	15.30	19.15	15.50	3.71	5.96
726	7.25	24.36	24.89	13.89	8.96	8.31

Table 22. Corticosterone concentrations (ng/ml) of stalled horses in relation to time relative to start of transport in Trial 3.

Animal	Pre-transport	Transport			Post-transport	
ID		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
53	3.50	4.77	4.20	3.57	1.23	1.18
63	7.06	13.70	10.69	9.23	36.16	8.29
66	2.59	8.24	8.11	11.27	8.19	10.57
72	48.15	37.56	56.64	44.65	8.72	20.56
310	8.32	26.36	34.30	42.12	3.92	47.47
612	8.91	14.40	11.99	9.16	10.69	3.14
614	13.21	28.38	15.62	18.45	7.30	4.55
705	3.00	10.40	17.41	26.95	6.87	5.63
720	3.34	10.77	13.98	11.87	4.40	4.05
723	2.72	12.95	11.59	12.57	5.97	5.37

Table 23. Corticosterone concentrations (ng/ml) of group horses in relation to time relative to start of transport in Trial 3.

Animal	Pre-transport	Transport			Post-transport	
ID		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
57	2.21	5.67	7.39	4.18	1.32	2.44
58	2.68	5.84	5.10	5.33	3.39	1.52
76	9.08	16.01	24.50	16.53	16.41	11.61
205	7.95	13.01	13.53	11.17	8.21	11.44
209	5.17	11.87	8.71	7.39	8.40	15.23
615	9.95	15.57	18.49	11.85	8.02	9.85
618	2.34	5.24	2.14	2.99	6.37	2.05
710	1.50	9.33	9.17	5.67	1.94	2.69
711	7.23	11.36	10.42	6.35	5.72	11.30
726	1.09	3.87	3.80	0.91	0.94	2.83

Table 24. DHEA concentrations (pg/ml) of stalled horses in relation to time relative to start of transport in Trial 3.

Animal	Pre-transport	Transport			Post-transport	
ID		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
53	1781	415	1479	262	402	171
63	1103	542	79	693	286	190
66	4742	637	719	2199	1675	1590
72	3461	1694	3780	1436	1234	782
310	4123	558	971	1894	3063	2487
612	1867	725	87	910	1193	899
614	1525	1125	343	1205	441	446
705	2471	725	1551	1205	682	586
720	607	209	457	795	592	1081
723	757	1334	782	1533	1118	1363

Table 25. DHEA concentrations (pg/ml) of group horses in relation to time relative to start of transport in Trial 3.

Animal	Pre-transport	Transport			Post-transport	
ID		2 hrs	4 hrs	6hrs	2 hrs	4 hrs
57	51	1444	308	662	210	446
58	1634	239	407	932	393	50
76	864	256	298	329	838	419
205	1153	987	1035	910	2647	1671
209	1815	419	1052	262	429	240
615	351	178	130	217	536	580
618	281	272	76	191	26	34
710	1129	859	1655	1655	1172	450
711	2089	1010	437	1524	1455	1995
726	342	1169	1084	940	2675	2877

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